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| (21) International Application Number: PCT/US90/00256 (22) International Filing Date: 10 January 1990 (10.01.90) (30) Priority data: 302,410 26 January 1989 (26.01.89) US (71) Applicant: DAMON BIOTECH, INC. [US/US]; 119 Fourth Avenue, Needham Heights, MA 02194 (US). (72) Inventors: TSANG, Wen-Ghih ; 15 Fairbanks Avenue, Lexington, MA 02137 (US). MAGEE, Andrew, S. ; 96 Tarbell Spring Road, Concord, MA 01742 (US). SHYR, Ann, W. ; 65 Oakmont Road, Newton Centre, MA 02159 (US). | | (74) Agents: LOREN, Ralph, A. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US). (81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i> |
| (54) Title: STABILIZATION OF AQUEOUS-BASED HYDROPHOBIC PROTEIN SOLUTIONS AND SUSTAINED RELEASE VEHICLE (57) Abstract <p>Disclosed is a method for producing stable, high concentration solutions of hydrophilic proteins. These methods are useful in producing vehicles which provide sustained release of proteins, e.g., hydrophobic proteins, into aqueous environments.</p> | | |

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STABILIZATION OF AQUEOUS-BASED HYDROPHOBIC
PROTEIN SOLUTIONS AND SUSTAINED RELEASE VEHICLE

Background of the Invention

The present invention relates to the
5 formation and stabilization of aqueous-based
solutions of hydrophobic proteins. Sustained release
vehicles made using these stabilized solutions are
also disclosed.

Much of the interest in identification,
10 genetic engineering and purification of proteins is
related to the possibility of in vivo use of the
proteins, e.g., as treatment for protein
deficiencies. Proteins such as enzymes and hormones
modulate reactions in the body and the lack, or an
15 insufficient amount, of these proteins leads to a
variety of deficiency problems. However, intravenous
or subcutaneous injections of protein into the system
are often insufficient for long term amelioration of
many problems because of toxicity and feedback
20 control problems unless relatively low levels are
used on a frequent basis. In order to solve these
problems and relieve patients, and potential
patients, of the onerous task of frequent injections,
a variety of different sustained release vehicles
25 have been tried. These vehicles are in two general
categories: those which release protein through a
breakdown mechanism, e.g., collagen or dextran

degradation by the body; and those which use some type of pump-type mechanism, either osmotic or electro-mechanical, to release material over time. The first class of sustained release vehicles have
5 potential problems with differential breakdown rates, thereby providing uneven release while the second class of vehicles are normally bio-incompatible objects which must be removed after exhausted, often surgically.

10 Among the more promising sustained release vehicles are the sodium alginate-based microcapsules described in United States Patent No. 4,690,682, filed September 1, 1987, on application of Dr. Franklin Lim, and United States Patent Application
15 Serial No. 121,214, filed November 16, 1987, on application of Wen-Ghih Tsang and Andrew Magee, both assigned to the assignee of the present application. These vehicles use tortuous path-like pores of sodium alginate microcapsules as a "filtering" device
20 whereby an osmotic gradient is set up between a high internal concentration of the material to be released and the large surrounding aqueous volume. The proteins or other materials which have been encapsulated in this type of vehicle have been
25 limited to hydrophilic materials which are easily dissolved in the aqueous solution used to make the capsules.

One odd phenomenon of alginate-protein solutions is the ability, over time, to form stable
30 two-phase solutions. Although other polymers form separate phases, these phases are sometimes unstable

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and/or denature the polymers. For example, Tolstoguzov, Antonov and their co-workers have shown that the casein-alginate-water and trypsin-alginate-water systems are useful for making protein spinneret fibers because of their ability to make two-phase solutions. However, these two-phase systems were investigated as alternatives for the denatured protein normally used to form these protein matrix fibers and denaturation was not considered a problem. Despite the ability to form stable two-phase systems, neither the casein nor trypsin system produced notably better results. The two-phase system formed is interesting, however, since both casein and trypsin are hydrophilic, easily soluble proteins.

Hydrophobic proteins, e.g., proteins which are substantially insoluble or have low solubilities in aqueous solutions, are particularly difficult to use in sustained release vehicles which dispense the protein into primarily aqueous solutions. There are several problems which contribute to this: first, it is difficult to obtain a meaningful concentration of hydrophobic proteins in the aqueous solution; second, to the extent that any concentration is obtained, it is relatively unstable; and third, there are surface effects at the interface between the phases.

None of the work done by the Tolstoguzov group appears to touch upon the problem of stabilizing hydrophobic protein systems. In fact, they did not report investigation of hydrophobic proteins in a two-phase system. Therefore, their

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work provides no clues to solve the need for sustained release vehicles to provide constant, time-controlled release of hydrophobic molecules.

Accordingly, an object of the invention is to provide a method stabilizing aqueous solutions of relatively high concentrations of hydrophobic proteins.

Another object of the invention is to provide a sustained release vehicle which is biocompatible and allows controlled release of proteins.

A further object of the invention is to provide sustained release systems usable for a broad variety of proteins, particularly hydrophobic proteins, without mechanical or electro-mechanical pumping systems.

These and other objects and features of the invention will be apparent from the following description.

20 Summary of the Invention

The present invention features methods of producing stable, high concentration aqueous solutions of hydrophobic proteins and sustained release vehicles made from those solutions. The invention is based, in part, on the discovery that aqueous solutions of certain polymers, e.g., alginic acid derivatives and other polysaccharides, will,

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when mixed with proteins, separate and form stable two-phase systems. This two-phase system can be turned into microcapsules which permits higher concentrations of proteins, e.g., hydrophobic proteins, to be encapsulated than is normally possible. Controlling the rate of release over time is also achievable using this system.

The method of producing the stable, high concentration aqueous solutions of the hydrophobic proteins commences with the formation of a first aqueous solution of the polymer which has the ability to form a two-phase systems when mixed with the protein, preferably an alginic acid derivative, e.g., sodium alginate. The hydrophobic protein is mixed or dissolved in the first polymeric solution, forming a polymer hydrophobic protein solution. An alginic acid derivative-hydrophobic protein solution is a preferred first solution. This solution is allowed time to stabilize, preferably at slightly above its freezing point with nutation, until two distinct phases form; one phase having a high concentration of the hydrophobic protein and the other having a lower concentration of the hydrophobic protein but richer in the polymer. The protein-rich phase is normally oily in consistency while the protein-poor phase is substantially aqueous. The phases may be separated and the protein-rich phase can provide a stable, high concentration aqueous solution of the hydrophobic protein. Preferred sodium alginate for use in this stabilization has a high mannuronic:guluronic acid ratio. Hydrophobic proteins useful in the invention include growth hormones such as those selected from a

group consisting of somatotropin, and derivatives and analogs thereof.

To make the sustained release vehicle of the invention, the same stabilization steps are followed. The sustained release vehicle can be made from the two-phases of the separated solution or in a preferred embodiment, from the protein-rich phase. The initial aqueous solution should be at or near protein saturation. After separation of the protein-rich phase, the alginate or other polysaccharide is gelled, e.g., by contacting the phase with a multivalent cation, thereby forming discrete gel balls. If sodium alginate is used, the preferred cations are calcium ions. The protein-rich phase forms pockets of protein in the gel ball.

The gel balls themselves may be used as sustained release vehicles but the formation of microcapsules from the gel balls is preferred. To form microcapsules, the gel balls are reacted with a membrane forming material, e.g., a polycationic material, thereby forming microcapsules with a protective membrane. The formed microcapsules may be further treated by putting a further protective coating thereon, e.g., by soaking the microcapsules in alginate solution to yield a negative surface charge. Preferred polycationic polymers are selected from a group consisting of polyornithine, polylysine, polyglutamic acid, and co-polymers, derivatives and mixtures thereof.

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The invention includes not just the method of making this sustained release vehicle and the stabilization method but also the sustained release vehicle itself, either in the gel ball or
5 microcapsule form. While any protein which forms the two-phase system with the polymer can be used, an alginate acid-growth hormone combination such as sodium alginate-somatotropin, is preferred.

Description of the Invention

10 The present invention permits the production of stable aqueous solutions of hydrophobic proteins, e.g., growth hormones, in higher concentrations than can otherwise be obtained. Further, stabilized, high concentration protein solutions can be formed into
15 sustained release vehicles which permit the protein to be released over time at a relatively steady controllable rate.

The invention is based on the production of the protein-rich oily phase of a two-phase polymeric hydrophobic protein solution. If alginic acid
20 derivatives are used as the polymer, this two-phase system does not appear immediately but rather develops over time. As will be evident from the following examples, the development and stabilization
25 of the two-phase system may take several days. The same alginic acid derivative-hydrophobic protein solution does not provide the same sustained release properties sought unless the two-phase system has developed.

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The following examples more clearly delineate the advantages and methods used in the invention.

Example 1.

5 This Example illustrates the solubilization and stabilization attributes of the two-phase system of the invention. Bovine somatotropin (bST) was added to both neutral saline and a 1.4% sodium alginate (Kelco LV) solutions. The bST was
10 substantially insoluble in saline, at pH 7.4. In contrast, a 50 mg/ml solution was prepared relatively easily in the sodium alginate system. When the sodium alginate bST solution was allowed to stand at 4°C. for forty hours with nutation, a two-phase
15 system developed. The alginate-rich phase, which was about 90% of the volume, had a protein concentration of about 20 mg/ml while the oily protein phase had a bST concentration of about 300 mg/ml, showing a pronounced concentration solubilization and
20 stabilization effect.

Example 2.

In this Example, the sustained release effect of the making microcapsules from the two-phase is compared with using an unseparated sodium
25 alginate-hydrophobic protein solution. A 1.4% (w/v) sodium alginate (Kelco LV) solution was prepared and bovine somatotropin (bST) was mixed into the alginate solution at a concentration of 50 mg/ml. As noted from the results of Example 1, this is a higher

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concentration then could be obtained without the alginate. One portion of the sodium alginate solution was encapsulated immediately, using standard techniques, by allowing drops of solution to fall
5 into a 1.2% calcium chloride solution, thereby forming gel balls. A jet-head droplet forming apparatus consisting of a housing having an upper air intake nozzle and an elongate hollow body friction fitted into a stopper. A syringe, e.g., a 10 cc
10 syringe, equipped with a stepping pump is mounted atop the housing with a needle, e.g., a 0.01 inch I.D. Teflon-coated needle, passing through the length of the housing. The interior of the housing is designed such that the tip of the needle is subjected
15 to a constant laminar air-flow which acts as an air knife. In use, the syringe full of the solution containing the material to be encapsulated is mounted atop the housing, and the stepping pump is activated to incrementally force drops of the solution to the
20 top of the needle. Each drop is "cut off" by the air stream and falls approximately 2.5-3.5 cm into an encapsulation solution containing 1.2% CaCl_2 and 0.3% 80/20 polyornithine/polyglutamic acid copolymer where it is immediately gelled and coated into
25 capsules.

The other portion of the sodium alginate-bST solution was held at 4°C. for forty hours while undergoing nutation or gentle mixing. After forty hours, the solution separated into two distinct
30 phases; an oily-phase containing most of the protein and a substantially aqueous phase containing most of the alginate. The entire solution containing the

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separated phases was used to make microcapsules, using the same procedure as previously described. A protein-rich phase acts as suspended pockets of high protein concentration, forming a visible spindle structure. Over time, the spindle structure disintegrates, releasing bST from the capsules.

The two sets of microcapsules were tested for sustained release by injection into Hypox rats. The rats' rate of growth was measured by weighing them every day. The rats which received the capsules made from the unseparated alginate-bST solution had a very rapid weight gain in days one and two and then a negative or substantially no weight gain thereafter, showing that all of the bST was released within two days. This is similar to the results for rats receiving a single large dosage injection of bST, which show a high initial weight gain followed by weight decreases or substantially flat weight gain after day two. In contrast, rats which received microcapsules made from the separated solution show a high weight gain in days one and two and then substantially constant weight gain for days two through seven. The sustained release results were similar to the results obtained by giving rats daily injections of bST, showing that the single injection of the microencapsulated bST acted as a reservoir, yielding a substantially continuous stream of bST to the rats.

The results with gel balls rather than formed microcapsules were not as good but still showed better results than the single injection form.

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Example 3.

In this Example, capsules made using the procedure previously described, including the nutation at 4° for forty hours, were perfused with Tris buffer to test sustained release characteristics. In vivo testing of bST formulations in Hypox rats, such as is described in Example 2, is limited in time duration due to an immune response by the animals after seven days. Therefore, perfusing experiments were performed in order to simulate the in vivo performance of these formulations over a longer time period.

The microcapsules were prepared using a 40 mg/ml bST solution in 1.6% Kelco LV sodium alginate. The solution was nutated for approximately forty hours at 4°C. and formed into microcapsules as described in Example 2. A 3% 80/20 polyornithine/polyglutamic acid copolymer was used for membrane formation.

Three different samples were used in the Example: a control group of capsules which was not perfused before injection, a first test group of microcapsules which were perfused for two days, and a second test group of microcapsules which were perfused for five days. Perfusion was carried out by flowing a Tris buffer, pH 7.4. at 37°C., past the capsules at a rate of 10 ml/hr.

After perfusion, the control and each of the test samples were injected into Hypox rats. Weight

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gain was measured as an indication of bST release rate. Table 1 shows the weight gain at day 2, days 2-4, days 4-7, and days 7-10.

TABLE 1

| 5 | <u>Average weight gain in grams</u> | | | |
|------------------|-------------------------------------|-----|-----|------|
| | Day 2 | 2-4 | 4-7 | 7-10 |
| Control | 13.0 | 7.2 | 1.7 | -1.1 |
| 2 days perfusion | 12.6 | 7.3 | 5.3 | -1.7 |
| 5 days perfusion | 14.3 | 5.3 | 4.3 | 0.5 |

10 As is clear from the results shown in Table 1, the samples which have been perfused for two or five days provide essentially identical growth rates and, therefore, release rates, as did the unperfused control sample. The performance of the partially
15 depleted samples suggest that the capsule formulation is capable of delivering bST at a steady state for significant time periods, exceeding seven days.

The foregoing Examples are meant to be non-limiting and are here solely for ease in
20 explanation of the invention. The invention is defined by the following claims.

What is claimed is:

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1. A method of producing sustained release of proteins comprising the steps of:

forming an aqueous solution of a polymeric material and a protein, said polymeric material
5 having the ability to form a two-phase system when mixed with said protein,

permitting said solution to separate into said two-phase system having a first phase containing a high concentration of said hydrophobic protein and
10 a second phase containing a low concentration of said hydrophobic protein, and

forming said sustained release vehicle from said solution, whereby said first phase forms pockets of protein within said vehicle.

15 2. The method of claim 1 wherein said polymeric material comprises a polysaccharide.

3. The method of claim 2 wherein said polysaccharide comprises an alginic acid derivative.

4. The method of claim 3 wherein said alginic
20 acid derivative comprises sodium alginate.

5. The method of claim 1 wherein said proteins comprise hydrophobic proteins.

6. The method of claim 5 wherein said aqueous solution is a substantially saturated solution of
25 said hydrophobic protein.

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7. The method of claim 1 wherein said separating step further comprises maintaining said aqueous solution at a temperature slightly above its freezing point until said two-phase system develops
5 and is stabilized.

8. The method of claim 7 wherein said solution undergoes nutation while being held at the temperature slightly above its freezing point.

9. The method of claim 4 wherein said step of
10 forming said sustained release vehicle comprises the step of gelling said first phase with a multivalent cation to form discrete gel balls.

10. The method of claim 9 wherein said step of forming said sustained release vehicle further
15 comprises the step of reacting said gel balls with a polycationic material to form a membrane about said gel balls, thereby forming microcapsules.

11. The method of claim 10 further comprising the step of putting a protective coating about said
20 microcapsules.

12. The method of claim 11 wherein said step of applying a protective coating comprises soaking said microcapsules in an alginate solution.

13. The method of claim 10 wherein said sodium
25 alginate is gelled by contact with calcium ions.

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14. The method of claim 12 wherein said polycationic polymer is selected from a group consisting of polyornithine, polylysine, and polyglutamic acid, and copolymers, derivatives, and mixtures thereof.

15. The method of claim 10 wherein said hydrophobic protein is a growth hormone.

16. The method of claim 15 wherein said growth hormone comprises somatotropin, a derivative, or an analog thereof.

17. The method of claim 3 wherein said alginic acid derivative has a high mannuronic:gluronic ratio.

18. The method of claim 9 wherein said method further comprises the step of separating said first phase from said second phase and forming said sustained release vehicle from said first phase.

19. A sustained release vehicle for providing in vivo sustained release of a hydrophobic protein, said sustained release vehicle comprising a cross-linked protein-rich phase of a mixture of a polymeric material and a protein, said polymeric material having the ability to form a two-phase system when mixed with said protein, said protein-rich phase being one of the phases of said two-phase system, said cross-linking being achieved by contacting said polymeric material with a cross-linking agent.

20. The sustained release vehicle of claim 19 wherein said polymeric material comprises a polysaccharide.
21. The sustained release vehicle of claim 20
5 wherein said polysaccharide comprises an alginic acid derivative.
22. The sustained release vehicle of claim 21 wherein said alginic acid derivative comprises sodium alginate.
- 10 23. The sustained release vehicle of claim 21 wherein said cross-linking agent comprises a multivalent ion.
24. The sustained release vehicle of claim 21 wherein said protein comprises a hydrophobic protein.
- 15 25. The sustained release vehicle of claim 24 wherein said mixture is a substantially saturated solution of said hydrophobic protein.
26. The sustained release vehicle of claim 19 wherein said phase separation takes place while
20 maintaining said mixture at a temperature slightly above its freezing point until said two-phase system develops and is stabilized.
27. The sustained release vehicle of claim 26 wherein said mixture undergoes nutation while being
25 held at the temperature slightly above its freezing point.

28. The sustained release vehicle of claim 23 wherein said separated protein-rich phase is in the form of pockets of protein contained within a gel ball after contacting with said multivalent ions.
- 5 29. The sustained release vehicle of claim 22 wherein said sodium alginate comprises sodium alginate with a high mannuronic:guluronic ratio.
30. The sustained release vehicle of claim 23 wherein said multivalent ion comprises a calcium ion.
- 10 31. The sustained release vehicle of claim 30 wherein said sustained release vehicle further comprises a membrane formed about said gel ball, said membrane formed by reacting said gel ball with a polycationic material.
- 15 32. The sustained release vehicle of claim 31 wherein said polycationic material is selected from a group consisting of polyornithine, polylysine, and polyglutamic acid, and mixtures, copolymers, and derivatives thereof.
- 20 33. A method of producing stable, high concentration aqueous solutions of hydrophobic proteins comprising the steps of:
- forming a first aqueous solution of an alginic acid derivative,
- 25 mixing said hydrophobic protein with said first alginic acid derivative solution, forming an alginic acid derivative-hydrophobic protein solution,

allowing two distinct phases form in said alginic acid derivative-hydrophobic protein solution, one containing a high concentration of said hydrophobic protein and one containing a low
5 concentration of said hydrophobic protein, and

separating said phase containing the high concentration of said hydrophobic protein to provide a stable, high concentration aqueous solution of said hydrophobic protein.

10 34. The method of claim 33 wherein said step of allowing said alginic acid derivative-hydrophobic solution to separate into two-phases is carried out at a temperature slightly above its freezing point.

35. The method of claim 33 wherein said alginic
15 acid derivative comprises sodium alginate.

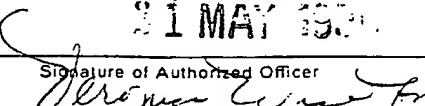
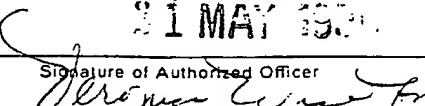
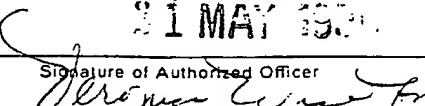
36. The method of claim 35 wherein said sodium alginate has a high mannuronic:guluronic acid ratio.

37. The method of claim 33 wherein said hydrophobic protein is a growth hormone.

20 38. The method of claim 39 wherein said growth hormone is selected from a group consisting of somatotropin, a derivative or an analog thereof.

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US90/00256**

| I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5) : A61K, 37/02; 37/36 U S C1 : 424/451,457; 514/12,54,964 | | | | | | | | | | | |
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| II. FIELDS SEARCHED <div style="text-align: center; margin-top: 5px;">Minimum Documentation Searched ⁷</div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%;">Classification System</th> <th style="width: 80%;">Classification Symbols</th> </tr> <tr> <td style="text-align: center; vertical-align: top;">U S</td> <td style="vertical-align: top;">424/451,457; 514/12,54,964</td> </tr> </table> <div style="text-align: center; margin-top: 5px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div> <div style="text-align: center; margin-top: 10px;">APS,CAS</div> | | | Classification System | Classification Symbols | U S | 424/451,457; 514/12,54,964 | | | | | |
| Classification System | Classification Symbols | | | | | | | | | | |
| U S | 424/451,457; 514/12,54,964 | | | | | | | | | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%;">Category [*]</th> <th style="width: 70%;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">US,A 4,690,682 (LIM) 01 September 1987 See Description, Columns 3-7.</td> <td style="text-align: center; vertical-align: top;">1-38</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">Chemical Abstracts, Volume 97, 1982 (Columbus, Ohio, USA), Antonov, Yu. A., See pages 466-467, Abstract No. 37761c.</td> <td style="text-align: center; vertical-align: top;">1-38</td> </tr> </table> | | | Category [*] | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ | Y | US,A 4,690,682 (LIM) 01 September 1987 See Description, Columns 3-7. | 1-38 | Y | Chemical Abstracts, Volume 97, 1982 (Columbus, Ohio, USA), Antonov, Yu. A., See pages 466-467, Abstract No. 37761c. | 1-38 |
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| Y | US,A 4,690,682 (LIM) 01 September 1987 See Description, Columns 3-7. | 1-38 | | | | | | | | | |
| Y | Chemical Abstracts, Volume 97, 1982 (Columbus, Ohio, USA), Antonov, Yu. A., See pages 466-467, Abstract No. 37761c. | 1-38 | | | | | | | | | |
| <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div> | | | | | | | | | | | |
| IV. CERTIFICATION <table style="width: 100%;"> <tr> <td style="width: 50%; vertical-align: top;"> Date of the Actual Completion of the International Search <div style="text-align: center;">03 May 1990</div> International Searching Authority <div style="text-align: center;">ISA/US</div> </td> <td style="width: 50%; vertical-align: top;"> Date of Mailing of this International Search Report <div style="text-align: center;">31 MAY 1990</div> Signature of Authorized Officer <div style="text-align: center;">  Fatemeh T. Moezie </div> </td> </tr> </table> | | | Date of the Actual Completion of the International Search <div style="text-align: center;">03 May 1990</div> International Searching Authority <div style="text-align: center;">ISA/US</div> | Date of Mailing of this International Search Report <div style="text-align: center;">31 MAY 1990</div> Signature of Authorized Officer <div style="text-align: center;">  Fatemeh T. Moezie </div> | | | | | | | |
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